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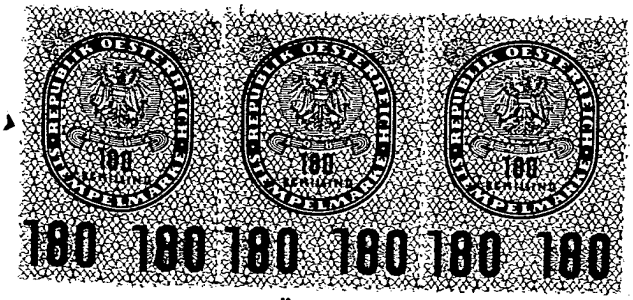

Aktenzeichen **A 1872/98**

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Das Österreichische Patentamt bestätigt, dass

die Firma IMMUNO Aktiengesellschaft in A-1221 Wien, Industriestraße 67,

am **10. November 1998** eine Patentanmeldung betreffend

"A Factor VIII polypeptide with Factor VIII:C activity",

überreicht hat und dass die beigeheftete Beschreibung samt Zeichnungen mit der ursprünglichen, zugleich mit dieser Patentanmeldung überreichten Beschreibung samt Zeichnungen übereinstimmt.

Österreichisches Patentamt
Wien, am 22. Oktober 1999

Der Präsident:

i. A.

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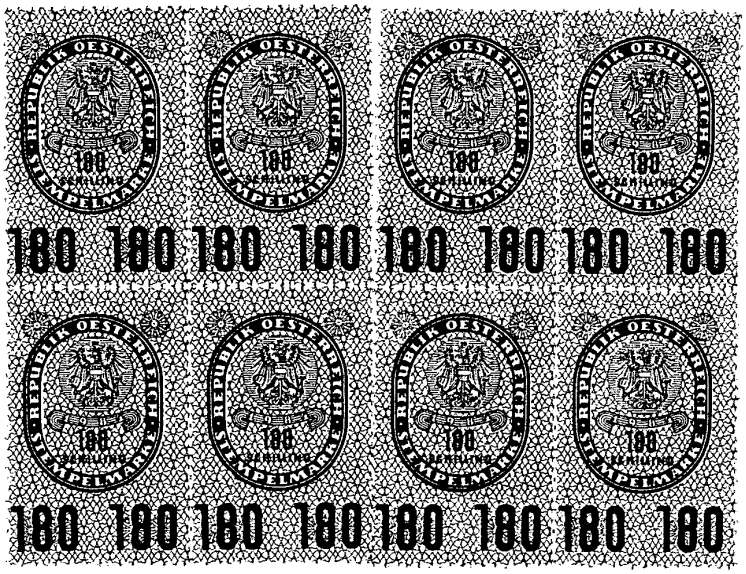

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The present invention relates to modified Factor VIII (FVIII) polypeptides with FVIII:C activity, having modifications in the A3 and/or C1 and/or C2 domain of the Factor VIII light chain sequence, nucleic acid molecules encoding such modified Factor VIII polypeptides, vectors and host cells comprising the nucleic acid molecules and compositions comprising the Factor VIII polypeptide for the treatment of bleeding disorders.

The arrest of bleeding involves the concerted action of various hemostatic pathways, which eventually lead to thrombus formation. Thrombi are depositions of blood constituents on the surface of the vessel wall, and are mainly composed of aggregated blood platelets and insoluble, cross-linked fibrin. Fibrin formation occurs by limited proteolysis of fibrinogen by the clotting enzyme thrombin. This enzyme is the final product of the coagulation cascade, a sequence of zymogen activations which occur at the surface of activated platelets and leukocytes, and of a variety of vascular cells (for review see K.G. Mann et al, Blood, 1990, vol. 76, pp. 1-16).

A key step in the coagulation cascade is the activation of factor X by the complex of activated factor IX (factor IXa) and activated Factor VIII (Factor VIIIa). Deficiency or dysfunction of the components of this complex is associated with the bleeding disorder known as hemophilia (J.E. Sadler & E.W. Davie: Hemophilia A, Hemophilia B, and von Willebrand's disease, in G. Stamatoyannopoulos et al. (eds): The molecular basis of blood diseases. W.B. Saunders Co., Philadelphia, 1987, pp. 576-602). Hemophilia A refers to deficiency in Factor VIII activity, whereas hemophilia B refers to factor IX deficiency. Current treatment consists of replacement therapy using pharmaceutical preparations consisting of the normal coagulation factor. Of these coagulation disorders, hemophilia A is the more frequent, affecting approximately 1 in 10,000 males. Replacement therapy of hemophilia A involves the repetitive administration of preparations comprising normal Factor VIII through intravenous infusion. The interval between infusions is imposed by the decay of Factor VIII activity in the circulation. The half-life of Factor VIII activity following infusion varies between individuals, and ranges between 10 and 30 hours. Thus,

prophylactic therapy requires infusion each two to three days. This poses a heavy burden on the life of hemophilia patients, especially when venous access has become difficult by local scar formation due to the frequent puncture by needles for intravenous infusion. It would be particularly advantageous if the frequency of infusion could be reduced by the use of Factor VIII with prolonged half-life. Half-life of Factor VIII may be prolonged by interfering in the mechanism of Factor VIII clearance, for instance by reducing the affinity of Factor VIII for receptors involved in its clearance, either directly by modifying Factor VIII in its binding site(s) for the clearance receptors involved, or indirectly, by using compounds which interfere in the interaction of Factor VIII with said receptors. The design of such agents, however, has been hampered until now by a lack of knowledge concerning the clearance mechanism of Factor VIII, cellular receptors involved in this process, and the molecular sites involved in Factor VIII-receptor interaction.

With regard to the mechanism of Factor VIII clearance limited knowledge exists on the molecular level. The Factor VIII protein is synthesized as a single chain polypeptide of 2332 amino acids, with the typical domain structure A1-A2-B-A3-C1-C2 (G.A. Vehar et al., Nature, vol. 312, 1984, pp. 337-342; J.J. Toole et al., Nature, vol. 312, 1984, 342-347). Due to intracellular endoproteolytic processing, Factor VIII enters into the circulation as a heterodimeric complex of heavy and light chain. The light chain comprises amino acid residues 1649-2332, and contains the A3-C1-C2 domains. The heavy chain contains the domains A1-A2-B (residues 1-1648) and is heterogeneous due to limited proteolysis at a number of positions within the B-domain. The Factor VIII heterodimer is devoid of biological activity, but becomes active as a cofactor of the enzyme factor IXa after proteolytic activation by thrombin or factor Xa. Proteolysis involves both the heavy- and light chain of Factor VIII (M.J.S.H. Donath et al., J. Biol. Chem., vol. 270, 1995, pp. 3648-3655), and results in cleavage of an amino-terminal fragment from the light chain, and of cleavage at the domain junctions in the heavy chain (between domains A1-A2 and A2-B). The activated cofactor is called Factor VIIIa and is a

heterotrimer consisting of the A1 domain, the A2-domain, and the light chain comprising the domains A3-C1-C2.

It is well known in the art that the half-life of the non-activated Factor VIII heterodimer is heavily dependent on the presence of von Willebrand factor, which has a high affinity for Factor VIII (but not Factor VIIIa) and serves as a carrier protein (J.E. Sadler and E.W. Davie: Hemophilia A, Hemophilia B, and von Willebrand's disease, in G. Stamatoyannopoulos et al. (eds): The molecular basis of blood diseases. W.B. Saunders Co., Philadelphia, 1987, pp 576-602). Patients with von Willebrand disease type 3, who have no detectable von Willebrand factor in their circulation, are known to display also a secondary deficiency of Factor VIII. Furthermore, the half-life of intravenously administered Factor VIII in these patients is 2 to 4 hours, which is considerably shorter than the 10 to 30 hours observed in hemophilia A patients.

These notions imply that Factor VIII is prone to rapid clearance from the circulation, and that this process is inhibited to some extent by complex formation with its natural carrier, von Willebrand factor. Nevertheless, half-life remains undesirably short.

Recently, a preliminary report has suggested that thrombin activated Factor VIII binds to Low Density Lipoprotein Receptor Protein ("LRP") (A. Yakhyaev et al., Blood, vol. 90 (suppl. 1), 1997, 126-I (abstract)). This abstract describes the cellular uptake and degradation of thrombin-activated Factor VIII fragments, and reports that the A2-domain, but not the other two subunits of the Factor VIIIa heterotrimer, interacts with cell-bound LRP. The authors propose that A2-domain binding to LRP further destabilizes the loose interaction of the A2 domain in the Factor VIIIa heterotrimer, and thus down-regulates Factor VIIIa activity.

LRP is known to be one of the receptors involved in the clearance of various proteins. LRP is also known in the art as the α2-Macroglobulin Receptor, and is a member of the Low Density Lipoprotein (LDL)-receptor family. It consists of two

non-covalently linked polypeptide chains: an α-chain (515 kDa) and a ß-chain (85 kDa) [for review see D.K. Strickland et al., FASEB J vol. 9, 1995, pp 890-898]. LRP is a multiligand receptor for lipoprotein and proteinase catabolism. The ß-chain contains a transmembrane domain and a short cytoplasmatic tail that is essential for endocytosis. The α-chain functions as a large ectodomain, and contains three types of repeats: epidermal growth factor-like domains, Tyr-Trp-Thr-Asp sequences and LDL-receptor class A domains. These class A domains are present in four distinct clusters, the socalled clusters I (2 domains), II (8 domains), III (20 domains) and IV (11 domains), which have been demonstrated to be involved in ligand binding.
LRP is expressed in a variety of tissues, particularly in placenta, lung, brain and liver. In the liver, LRP is present on parenchymal cells and Kupffer cells. In addition, LRP is expressed in an array of cell types, like fibroblasts, smooth muscle cells, Leydig- and Sertoli cells, and monocytes. Differentiation of monocytes to macrophages is associated with a dramatic increase in the expression of LRP. Finally, LRP is also expressed in cell types like monkey kidney (COS) cells or chinese hamster ovary (CHO) cells (D.J. FitzGerald et al., J. Cell Biol. vol. 129, 1995, pp 1533-1541), both of which are frequently used for the expression of mammalian proteins, including Factor VIII (R.J. Kaufman et al., Blood Coag. Fibrinol. vol. 8 (suppl. 2), 1997, pp 3-14).

LRP is involved in the clearance of a variety of ligands, including proteases, Kunitz-type inhibitors, protease-serpin complexes, lipases and lipoproteins, suggesting that LRP serves an essential role in various physiological and pathophysiological clearance processes (Narita et al., Blood, vol. 2, pp. 555-560, 1998; Orth et al., Proc. Natl. Acad. Sci., vol. 89, pp. 7422-7426, 1992; Kounnas et al., J. Biol. Chem., vol. 271, pp. 6523-6529, 1996). Its physiological importance is particularly apparent from the notion that LRP-knock out mice do not survive the embryonic stage (Herz, J. Curr. Opin. Lipidol. vol. 4, 1993, pp. 107-113). The secretion of LRP may be complicated by the fact that it interacts with multiple ligands. However, within the cell LRP is associated with its chaperone protein Receptor Associated Protein (RAP). When bound to RAP,

LRP is unable to interact with any of its known ligands (Herz et al., J. Biol. Chem., vol. 266, pp. 21232-21238, 1991).

The interaction of LRP with its natural ligands can effectively be blocked by soluble LRP fragments. These may be obtained by a variety of methods known in the art including recombinant techniques, and as such provide access to effective LRP antagonists (I.R. Horn, J. Biol. Chem., vol. 272, 1997, pp. 13608-13613; B. Vash et al., Blood, vol. 92, 1998, pp. 3277-3285).

In view of the typical role of LRP in the clearance of proteases, inhibitors, and protease-inhibitor complexes, and it can be noted that LRP also binds the activated, non-enzymatic cofactor Factor VIIIa (A. Yakhyaev et al., Blood vol. 90 (suppl. 1), 1997, 126-I (abstract)). While this disclosure suggests a role of LRP in Factor VIIIa regulation, it lacks any hint to a role in LRP regulation of non-activated, heterodimeric Factor VIII, although this would be of potential interest for clearance of Factor VIII from the circulation, and thus for Factor VIII half-life.

There have been several attempts in prior art to improve the pharmacokinetic profiles of Factor VIII including modifications in various regions of the VIII polypeptides:
WO 87/07144 describes various modifications in proteolytic cleavage sites encompassing arginine and lysine residues to reduce the lability of the molecules for specific protease-catalyzed cleavage, for example, the Factor Xa cleavage site between Arg 1721 and Ala 1722.
WO 95/18827, WO 95/18828 and WO 95/18829 describe Factor VIII derivatives comprising modifications in the A2-region of the heavy chain.
WO 97/03193 discloses Factor VIII polypeptide analogs wherein the modifications comprise the alteration of the metal-binding properties of the molecule.
In WO 97/03195 Factor VIII:c polypeptide analogs are described, wherein modifications at one or more amino acid residues adjacent an Arg residue are provided.

EP 0 808 901 describes the construction of Factor VIII variants having at least one mutation in at least one immunodominant region of Factor VIII and the use of this Factor VIII variant for the treatment of patients with Factor VIII inhibitors. These modifications do not lead to a prolonged half life or increased stability, either in vivo or in vitro of the Factor VIII variant.

In view of the prior art, none of the documents disclose any indication that modification in the Factor VIII light chain leads to a modified binding affinity to a cellular receptor and, as a result, to a decreased clearance of the Factor VIII protein and to prolonged half life and increased stability of the Factor VIII.

It is therefore an object of the present invention to provide a Factor VIII polypeptide with Factor VIII:C activity having increased half-life and/or increased stability of the Factor VIII protein *in vivo* and/or *in vitro*.

In accordance with the object the present invention provides a Factor VIII polypeptide having a modification in the light chain of the molecule which affects the binding affinity to LRP.

In a preferred embodiment of the invention, the modification is in the A3 domain, between AA 1690 and 2032, in the C1 domain, between AA 2033 and 2172 and/or in the C2 domain of the light chain, between AA 2173 and 2332 (all amino acid numerations made in the present application with respect to the Factor VIII sequence refer to the numbering of Vehar et al. (Nature, vol. 312, 1984, pp 337-342), which is hereby incorporated by reference.

More specifically, the modification is in the A3 domain between AA 1711 (Met) and 1725 (Gly), AA 1743 (Phe) and 1749 (Arg), AA 1888 (Ser) and 1919 (His), AA 1942 (Trp) and 1947 (Met) and/or AA 1959 (Ser) and 1974 (Ala).

In a further embodiment of the present invention, the modification is in the C1 domain between AA 2037 (Ile) and 2062

(Trp), AA 2108 (Asp) and 2118 (Asn) and/or AA 2154 (Thr) and 2158 (Ile). More preferred, the modification is between AA 2112 Trp) and 2115 (Tyr).

Preferably, the modification is between AA 2209 (Arg) and 2234 (Phe) and/or AA 2269 (His) and 2281 (Lys) of the C2 domain.

More preferably, the modification is between AA 2211 (His) and 2230 (Leu).

In the course of the present invention it has been found that inhibition of LRP by its antagonist, RAP, results in accumulation of Factor VIII light chain in the medium. This demonstrates that cellular uptake of Factor VIII heterodimer involves a LRP-dependent mechanism.

Surprisingly, it has been found that a modification in the light chain of the Factor VIII polypeptide leads to a similar effect, that is increased half life and stability of the Factor VIII protein. Due to the modification in the Factor VIII molecule the binding affinity to LRP decreases and the rapid clearance of the protein is inhibited. This finding offers new opportunities for the improved treatment of coagulation disorders and additionally, which might be necessary in the preparation of the Factor VIII compositions.

Due to the modification in the Factor VIII polypeptide, the increase of the in vivo and in vitro half life of the Factor VIII molecule according to the present invention can be at least 10%, preferably 25%, more preferably 50%, more preferably 90% in comparison to wild-type Factor VIII protein.

Said Factor VIII polypeptides or Factor VIII variants according to the present invention exert their beneficial effect because they are representing interactive regions ("exosites") located on the subunits of the Factor VIII heterodimer, in particular on the Factor VIII light chain (domains A3-C1-C2). The term "exosite" is used herein in a broad sense, and refers to relatively hydrophilic parts of the protein which are likely to be oriented towards the surface of the Factor VIII molecule

(Kyte and Doolittle, J. Mol. Biol., vol. 157, pp. 105-132, 1982).

Although this method according to Kyte and Doolittle, employs principles that have already been appreciated in the art, and is based on the Factor VIII sequence as previously published, these hydrophilic exosites have previously received virtually no attention.

For example, the exosite at AA Ser 1784 to Asp 1831 includes the binding region of Factor IX already described in literature (AA 1801 to 1823, P.J. Lenting et al., J. Biol. Chem., vol. 271, pp. 1935-1940). This is a clear indication of the relevance of the hydropathy plots used for the identification of the exosites. The term "binding site" refers herein to a typical sequence pattern of amino acids, including natural and synthetic analogs thereof, which comprise the minimal requirements for binding of non-activated Factor VIII to LRP.

In a first set of particularly preferred embodiments of the invention, the polypeptide has a modification in one or more of the exosites within the sequence of the Factor VIII polypeptide, preferably of the Factor VIII light chain, and more preferably of the Factor VIII C2-domain. Furthermore, although the invention covers binding sites based on exosites of Factor VIII from any mammalian species, said polypeptides are preferably derived from the sequence of human Factor VIII.

The modification can be carried out by, for instance, directed in vitro mutagenesis or PCR or other methods of genetic engineering known from the state of the art which are suitable for specifically changing a DNA sequence for directed exchanges of amino acids (Current Protocols in Molecular Biology, vol. 1, ch. 8 (Ausubel et al. eds., J. Wiley and Sons, 1989 & Supp. 1990-93); Protein Engineering (Oxender & Fox eds., A. Liss, Inc. 1987)). Said modification can be a mutation, deletion or insertion in the region of the Factor VIII light chain.

The present invention further provides the nucleic acid which encodes any of the modified Factor VIII proteins covered by the

present invention. The nucleic acid may be DNA or RNA. The nucleic acid is contained in an expression vector that provides the appropriate elements for the expression of said DNA or RNA. The expression vector may comprise, for example, in the direction of transcription, a transcriptional regulatory region and a translational initiation region functional in a host cell, a DNA sequence encoding for the FVIII polynucleotide of the present invention and translational and transcriptional termination regions functional in said host cell, wherein expression of said nucleic sequence is regulated by said initiation and termination regions. The expression vector may also contain elements for the replication of said DNA or RNA. The expression vector may be a DNA or an RNA vector. Examples for DNA expression vectors are pBPV, pSVL, pRc/CMV, pRc/RSV, myogenic vector systems (WO 93/09236) or vectors derived from viral systems, for example from vaccinia virus, adenoviruses, adeno-associated virus, herpesviruses, retroviruses or baculo viruses. Examples for RNA expression vectors are vectors derived from RNA viruses like retroviruses or flaviviruses.

For some specific applications in gene therapy, i. e. when the nucleic acid per se is injected into an organ of a mammal, the nucleic acid, DNA as well as RNA, may be chemically modified. The chemical modifications may be modifications that protect the nucleic acid from nuclease digest, for example by stabilizing the backbone or the termini.

The expression vector containing the nucleic acid which encodes the modified Factor VIII polypeptide according to the present invention can be used to transform host cells which then produce said polypeptide. The transformed host cells can be grown in a cell culture system to *in vitro* produce said polypeptide. The host cells may excrete the modified Factor VIII polypeptide into the cell culture medium from which it can be prepared. The host cells may also keep the modified Factor VIII polypeptide inside their cell walls and the hybrid protein may be prepared from the host cells.

The host cells may be cells derived from the body of a mammal, for example fibroblasts, keratinocytes, hematopoietic cells,

hepatocytes or myoblasts, which are *in vitro* transformed with an expression vector system carrying a nucleic acid according to the present invention and re-implanted into the mammal. The Factor VIII polypeptide encoded by said nucleic acid will be synthesized by these cells *in vivo* and they will exhibit a desired biological activity in the mammal. In one specific embodiment, the mammal is a human patient suffering from hemophilia.

The nucleic acid encoding the modified Factor VIII polypeptide according to the present invention, may also be used to generate transgenic animals, which express said modified Factor VIII polypeptide proteins *in vivo*. In one embodiment of this specific application, the transgenic animals may express the Factor VIII polypeptide in endogenous glands, for example in mammary glands from which the said proteins are secreted. In the case of the mammary glands, said Factor VIII proteins are secreted into the milk of the animals from which said proteins can be prepared. The animals may be mice, cattle, pigs, goats, sheep, rabbits or any other economically useful animal.

The expression vector containing the nucleic acid which encodes any Factor VIII polypeptide that is covered by the present invention can further be administered to a mammal without prior *in vitro* transformation into host cells. The practical background for this type of gene therapy is disclosed in several patent applications, for example in WO 90/11092. The expression vector containing said nucleic acid is mixed with an appropriate carrier, for example a physiological buffer solution and injected into an organ, preferably a skeletal muscle, the skin or the liver of a mammal. The mammal is preferably a human and more preferably a human suffering from a genetic defect, most preferably the human is suffering from a blood clotting disorder. In one specific embodiment, the mammal is a human patient suffering from hemophilia and the nucleic acid that is contained in the expression vector encodes the modified Factor VIII polypeptide as described.

It is advantageous that the modified Factor VIII protein according to the present invention exhibits a Factor VIII

procoagulant activity of at least 50%, more preferably at least 80%, particularly at least 100%, of the Factor VIII procoagulant activity of a Factor VIII protein without the modification leading to decreased binding affinity to LRP, for example, of commercially available Factor VIII preparation based on recombinant or plasmatic Factor VIII:C.

The evaluation of the Factor VIII procoagulant activity can be performed by any suitable test, especially by those tests which are routinely carried out when assaying Factor VIII samples, like the one stage clotting assay, as described e.g. in Mikaelsson and Oswaldson, Scand. J. Haematol. Suppl. 33, pp. 79-86, 1984 or a chromogenic assay such as Factor VIII IMMUNOCHROM (Immuno).

Factor VIII activity may also be assessed by measuring the ability of Factor VIII to function as a cofactor for Factor IXa in the conversion of Factor X to Factor Xa employing a chromogenic substrate for Factor Xa (Coatest Factor VIII, Chromogenix, Moelndal, Sweden). In addition other assays that serve to determine the amount of Factor VIII activity in a sample may be utilized to test the Factor VIII activity of the modified proteins that are described in the present invention.

The actual assay whether any new modified Factor VIII protein exhibits a certain percentage of Factor VIII procoagulant activity is preferably carried out in parallel with an assay for the same Factor VIII molecule without the modification in the LRP-binding domain (e.g. Factor VIII wild type or a fully active B-domain deleted Factor VIII). With such a calibrated assay of the mutated Factor VIII molecule, the relative procoagulant activity (the percentage of activity compared to 100% activity of the wild type or the B-domain deleted Factor VIII) may be assayed without the risk of environmental error. Since with in vitro-tests for Factor VIII procoagulant activity, the results may often be affected with errors due to their artificial design, both properties are preferably also assayed by in vivo or ex vivo tests to obtain more reliable results with respect to the activity values.

As with the in vitro assays, parallel testing of the Factor VIII molecule without the modification is also preferred when performing the in vivo tests. Suitable animal models for evaluating the Factor VIII:C activity are described by WO 95/01570 and EP 0 747 060.

The preparation according to the present invention can be provided as a pharmaceutical preparation having modified Factor VIII polypeptide according to the present invention as a single component preparation or in combination with other components as a multiple component system. In a special embodiment, Factor VIII proteins or the modified Factor VIII molecules according to the invention can be combined with one or more polypeptides that selectively inhibit binding and internalisation of Factor VIII by low density lipoprotein receptor-related protein (LRP), wherein the polypeptide is RAP.

According to another object the present invention provides a composition comprising a Factor VIII molecule and one or more polypeptides that antagonistically interfere in the interaction between Factor VIII and LRP and therefore selectively inhibit binding and internalisation of Factor VIII by low density lipoprotein receptor-related protein (LRP). Preferably, this polypeptide is RAP or a soluble LRP fragment with an antagonistic effect. Preferably, the soluble LRP fragment binds to the FVIII in the FVIII-LRP binding region.

Said preparations may be used as the active component of pharmaceutical compositions for treating patients suffering from genetic disorders, preferably coagulation disorders and most preferably hemophilia, for example hemophilia A. Said compounds may further be used as the active component of pharmaceutical compositions for treating patients suffering from temporary disorders in their thrombotic or fibrinolytic systems, for example before, during or after surgery.

According to the present invention, a pharmaceutical composition is meant to be administered to mammals, preferably humans. In preparing the pharmaceutical product, the compounds of the

present invention, the modified Factor VIII polypeptide, the nucleic acids encoding it or transformed cells which are capable of expressing it *in vivo*, are mixed with physiologically acceptable carriers.

The compositions disclosed in the present invention may be formulated for administration in any convenient way, and the invention includes within its scope pharmaceutical compositions containing a therapeutically effective amount of Factor VIII. Such compositions may be formulated in a conventional manner using one or more pharmaceutically acceptable carriers or excipients. Suitable carriers include, but are not limited to, diluents or fillers, sterile aqueous media and various non-toxic organic solvents. The compositions may be formulated in the form of powders, aqueous suspensions or solutions, injectable solutions and the like. Appropriate dosage forms may be easily identified by a person skilled in the art.

The method of treatment of coagulation disorders according to the present invention should be carried out employing a dosage regimen which ensures maximum therapeutic response until improvement is achieved, and thereafter the minimum effective level which gives appropriate protection against bleeding. The dosage for intravenous administration may vary between approximately 10 and 300 IU/kg body weight, preferably between approximately 10 and 100 IU/kg body weight and more preferably between 20 and 40 IU/kg body weight. Appropriate dosage may also be dependent on the patient's general health, age and other factors which may influence the response to the drug. The drug may be administered by continuous infusion, or at regular intervals of to maintain the therapeutic effect at the desired level.

Another aspect of the invention relates to a method for the preparation of modified Factor VIII molecules according to the invention having a modification in the light chain. The sequence coding for the modified Factor VIII molecule is inserted into an appropriate expression system, for example, an expression vector, and appropriate cells are transfected with the recombinant DNA. Preferably, permanent cell lines are

established which express the modified Factor VIII. The cells are cultivated under optimal conditions for gene expression, and modified Factor VIII is isolated either from a cell culture extract or from the cell culture supernatant. The recombinant molecule can be further purified by all known chromatographic methods, such as anion or cation exchange, affinity or immunoaffinity chromatography or a combination thereof.

The modified Factor VIII is preferably produced by recombinant expression. They can be prepared by means of genetic engineering with any usual expression systems, such as, for instance, permanent cell lines or viral expression systems. Permanent cell lines are prepared by stable integration of the extraneous DNA into the host cell genome of e.g. vero, MRC5, CHO, BHK, 293, Sk-Hep1, particularly liver and kidney cells, fibroblasts, keratinocytes or myoblasts, hepatocytes or stem cells, for example hematopoietic stem cells, or by an episomal vector derived, e.g. from papilloma virus. Viral expression systems, such as, for instance, the vaccinia virus, baculovirus or retroviral systems, can also be employed. As cell lines, vero, MRC5, CHO, BHK, 293, Sk-Hep1, gland, liver and kidney cells are generally used. As eukaryotic expression systems, yeasts, endogenous glands (e.g. glands of transgenic animals) and other types of cells can be used, too. Of course, transgenic animals can also be used for the expression of the polypeptides according to the invention or derivatives thereof. For the expression of recombinant proteins, CHO-DHFR-cells have proved particularly useful (Urlaub et al., Proc.Natl.Acad.Sci., USA, vol 77, pp. 4216-4220, 1980).

For the recombinant preparation of modified Factor VIII according to the present invention, prokaryotic expression systems can be used, too. Systems, allowing expression in E.coli or B. subtilis are particularly useful.

The Factor VIII polypeptide according to the present invention is expressed in the respective expression systems under the control of a suitable promoter. For expression in eukaryotes, all known promoters are suitable, such as SV40, CMV, RSV, HSV,

EBV, ß-actin, hGH or inducible promoters, such as, for instance, hsp or metallothionein promoter.

According to the present invention a full length Factor VIII cDNA as well as any derivatives thereof, having Factor VIII:C activity, (f.e. B-domain deleted Factor VIII mutants, F VIII mutants which have partially deleted B-domains) can be used as starting material for the construction of the modified Factor VIII polypeptide. It can originate from any mammalian species, preferably from human, porcine or bovine sources.

The invention is illustrated in the subsequently described examples. While exemplary of the present invention as applied to the identification, preparation and use of improved compositions with reduced binding of the Factor VIII light chain to LRP, the invention is construed to be applicable to LRP-binding of the Factor VIII heavy chain as well. Variations within the purview of one skilled in the art are to be considered to fall within the scope of the present invention. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those describes herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

The following examples and the figures illustrate the present invention, they, however, do not limit the scope of the invention in any way.

Figure 1 shows the interaction between Factor VIII (panel A), thrombin-activated Factor VIII (Factor VIII, panel B), Factor VIII heavy chain (panel C) or Factor VIII light chain (panel D) and immobilized LRP employing surface plasmon resonance analysis. Details are provided in Example I. Comparison of panels A to D shows that Factor VIII, thrombin-activated Factor VIII, and Factor VIII light chain, but not Factor VIII heavy chain efficiently interact with LRP.

Figure 2 shows that Factor VIII light chain binds to immobilized LRP in a reversible and dose-dependent manner. Kinetic parameters for this interaction have been summarized in Table II, which is presented in Example II. Binding was assessed as described in Example II.

Figure 3 shows the effect of the LRP-antagonist RAP on the concentration of Factor VIII light chain in medium of Factor VIII light chain expressing cells. The experiments have been performed as described in Example III. In the absence of RAP (open symbols), the rise of Factor VIII light chain in medium is less than in its presence (closed symbols).

Figure 4 shows the effect of the LRP antagonist RAP on the concentration of the intact Factor VIII heterodimer in medium of Factor VIII expressing cells. Experimental details have been described in Example IV. In the presence of RAP (open symbols), the rise in Factor VIII activity is less than in its presence (closed symbols).

Figure 5a and b show hydropathy plots of Factor VIII light chain domains of A3, C1 and C2. The plot was constructed as described in Example VI. The plot reveals the existence of various discrete regions with low hydropathy values, which reflect the hydrophilic nature associated with potentially exposed exosites. These are indicated as A to K (Fig. 5a) and I to IV (Fig. 5b).

Figure 6 displays the interaction of Factor VIII C2 domain with immobilized LRP in the presence of the C2-domain directed antibody ESH-8. Binding has been analyzed employing surface plasmon resonance as described in Example VII. In the absence of C2 domain, ESH-8 displays no significant binding on immobilized LRP. In the presence of C2 domain, however, a dose dependent increase in binding to LRP is observed. This demonstrates that Factor VIII C2 domain binds to LRP.

Examples

Example I: The Factor VIII light chain comprises a LRP binding site

The binding of Factor VIII and subunits thereof to Low Density Lipoprotein Receptor-related Protein (LRP) was investigated employing purified components. LRP, Factor VIII, Factor VIII light chain, Factor VIII heavy chain, thrombin-activated Factor VIII were obtained employing previously established methods (Moestrup S.K. et al., J. Biol. Chem., vol. 266, 1991, pp. 14011-14017; Lenting P.J. et al., J. Biol. Chem., vol. 269, 1994, pp. 7150-7155; Curtis J.E. et al., J. Biol. Chem., vol. 269, 1994, pp. 6246-6250, respectively).

The interaction with LRP was examined employing surface plasmon resonance (SPR) analysis at a BIAcore™2000 biosensor system (Pharmacia Biosensor AB, Uppsala, Sweden). LRP was immobilized onto a CM5 sensorchip, using the amine coupling kit according to the instructions of the supplier (Pharmacia Biosensor, Uppsala, Sweden), at a concentration of 8.3 fmole/mm^2. A control channel on the sensorchip was activated and blocked, using amine coupling reagents without immobilization of protein.

Factor VIII or derivatives thereof at a concentration of 100 nM were passed over the control channel to asses nonspecific binding, and over the LRP coated channel in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ and 0.005 % (v/v) Tween-20 with a flow of 5 ml/min for a 2 min period at 25°C. The association between the various proteins and LRP is shown in Figure 1, and is expressed in terms of Resonance Units. In Table 1, the maximal increase in Resonance Units for the various derivatives is summarized. These data demonstrate that Factor VIII, thrombin-activated Factor VIII and Factor VIII light chain are able to interact with LRP. In contrast, Factor VIII heavy chain was unable to bind LRP. It is evident therefore, that the binding moiety of Factor VIII or thrombin-activated Factor VIII for LRP is located within the A3-C1-C2 region (residues 1690-2332).

Table 1: Binding of Factor VIII and its subunits to immobilized LRP as detected employing SPR-analysis. Binding to LRP is expressed as Resonance Units, and is corrected for nonspecific binding.

Protein	Binding (Resonance Units)
Factor VIII	262
Factor VIII heavy chain	0
Factor VIII light chain	305
Factor VIII thrombin-activated	446

Example II: Association kinetics of immobilized LRP and Factor VIII light chain

The kinetic parameters for the interaction between Factor VIII light chain and immobilized LRP were determined employing SPR-analysis at a BIAcore™2000 biosensor system (Pharmacia Biosensor AB, Uppsala, Sweden). This method is generallly known in the art, and has for example been applied to the kinetic analysis of the interaction between LRP and Receptor Associated Protein (RAP) (Horn I, in LRP-ligand interactions: kinetics and structural requirements; PH.D.-thesis, 1997, pp. 65-106, University of Amsterdam). LRP was immobilized onto three channels of a CM5-sensorchip as described in Example I at a concentration of 6.7 fmole/mm^2. A control channel to assess nonspecific binding was prepared as described in example I. Various concentrations of Factor VIII light chain (150, 175, 200, 225 and 250 nM) were passed over the control channel and over the LRP coated channels in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ and 0.005 % (v/v) Tween-20 with a flow of 20 ml/min for a 2 min period at 25°C to allow association to occur. Subsequently, the channels were incubated with the same buffer at similar flow to allow dissociation to occur. As shown in fig. 2, a dose-dependent association and dissociation curve is observed.

Data were analyzed by Biacore Evaluation Software (Pharmacia Biosensor, Uppsala, Sweden). Data analysis revealed a best fit for the interaction between Factor VIII light chain with two classes of binding sites. Subsequently, association and dissociation rate constants (k_{on} and k_{off}, respectively) for both binding sites were calculated. These rate constants were subsequently used to obtain the affinity constants (K_d) for these interactions.

Table II: Rate constants for the interaction between Factor VIII light chain and immobilized LRP. Data analysis indicate the interaction of Factor VIII light chain with two classes of binding sites, which are represented as A and B, respectively.

Class	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (= k_{off}/k_{on}; nM)
A	3.0×10^5	5.5×10^{-2}	182
B	7.2×10^4	2.7×10^{-3}	37

Example III: Interaction between Factor VIII light chain and cell-bound LRP

As Factor VIII light chain efficiently binds to LRP in a system employing purified components, we investigated the interaction between Factor VIII light chain and LRP which is expressed at the surface of living cells. In order to express the light chain of Factor VIII, *i.e.* residues 1649 to 2332 (Toole J.J. et al., Nature, vol. 312, 1984, pp. 342-347), a construct was prepared encoding the Factor VIII signal peptide fused to residues 1649 to 2332. This construct was prepared as follows. The previously described plasmid pBPV-Factor VIII-dB695 (K. Mertens et al., Brit. J. Haematol. ,vol. 85, 1993, pp. 133-142) was used as template for the construction of two Factor VIII fragments employing the polymerase chain reaction (PCR). One fragment was made by using the sense-primer A1 (5'-TTA GGA TCC ACC ACT ATG CAA ATA GAG CTC TCC-3') containing a BamH1 recognition site and a part encoding N-terminal residues of the Factor VIII signal peptide, and the anti-sense primer A-1 (5'-AGT AGT ACG AGT TAT

TTC ACT AAA GCA GAA TCG C-3') encoding C-terminal residues of the Factor VIII signal peptide and N-terminal residues of Factor VIII light chain. A second fragment was made by using the sense primer B1 (5'-TTG CGA TTC TGC TTT AGT GAA ATA ACT CGT ACT AC-3') encoding C-terminal residues of the Factor VIII signal peptide and N-terminal residues of Factor VIII light chain, and the anti-sense primer B-1 (5'-ATT GCG GCC GCT CAG TAG AGG TCC TGT GCC TC-3') containing a Not1 recognition site, a stop codon and a part encoding C-terminal residues of the Factor VIII light chain. In a second reaction, the products of both reactions were used as a template for the construction of the final fragment designated Factor VIII-SPLC, employing primers A1 and B-1. Factor VIII-SPLC consisted of a BamH1 recognition site, a part encoding the Factor VIII signal peptide fused to a part encoding Factor VIII light chain, a stop codon and a Not1 recognition site. Factor VIII-SPLC was subsequently digested with BamH1 and Not1 and ligated into the expression vector pcDNA3.1 (Invitrogen, Leek, The Netherlands) which was digested employing the same restriction enzymes. The resulting vector designated pcFactor VIII-LC was transfected to chinese hamster ovary K1 (CHO-K1) cells (ATCC CCL-61) employing calcium phosphate precipitation (J. Sambrook et al., Molecular Cloning; A laboratory manual, Cold Spring Laboratory Press, Cold Spring Harbor, U.S.A., 1989, p. 1637). CHO-K1 cells have been established to express LRP constitutively at their cell surface (D.J. FitzGerald et al., J. Cell. Biol., vol. 129, 1995, pp. 1533-1541). CHO-K1 cells stably expressing were obtained under selection with G-148 (Gibco-BRL, Breda, The Netherlands) at a concentration of 800 µg/ml.

CHO-K1 cells stably expressing Factor VIII light chain were grown to confluency in 2 wells of a 6-well plate (Nunc A/S, Roskilde, Denmark). The wells were washed five times employing Dulbecco's Modified Eagle's Medium-F12 Medium (DMEM-F12) (Gibco-BRL, Breda, The Netherlands), and 1 ml of DMEM-F12 was added. In one of the wells, the LRP-antagonist RAP was added to a concentration of 20 mg/ml immediately and at 2 and 4 hours after washing of the cells. Samples were drawn up to six hours after washing the cells, and subsequently analyzed for the concentration of Factor VIII light chain using a method known in

the art (Lenting P.J. et al., J. Biol. Chem., vol. 269, 1994, pp. 7150-7155). As shown in Fig. 3, the concentration of Factor VIII light chain in medium increased in time in the absence of RAP. In the presence of RAP, however, the extent of Factor VIII light chain rise is increased compared to the absence of RAP. Thus, inhibition of LRP is associated with an accumulation of Factor VIII light chain in medium. This clearly demonstrates that the cellular uptake of Factor VIII light chain involves a LRP-dependent mechanism.

Example IV: Interaction between Factor VIII and cell-surface exposed Low Density Lipoprotein Receptor-related Protein

As described in example III, Factor VIII light chain interacts with cell-surface exposed LRP. We therefore also investigated whether the intact Factor VIII protein interacts with cell-surface exposed LRP. A previously established mouse fibroblast cell line which was stably transfected in order to produce Factor VIII (Mertens K. et al., Brit. J. Haematol., vol. 85, 1993, 133-142) was grown to confluency in 2 wells of a 6-well plate (Nunc A/S, Roskilde, Denmark). The cells were washed five times employing Iscove's Modified Eagle's Medium (IMEM) (Boehringer Ingelheim/Biowhitaker, Verviers, Belgium), and 1 ml of IMEM was added. In one of the wells, the LRP-antagonist RAP was added to a concentration of 20 mg/ml immediately and at 2 and 4 hours after washing of the cells. Samples were drawn up to six hours after washing the cells, and subsequently analyzed for Factor VIII cofactor activity employing a previously established method (Mertens K. et al., Brit. J. Haematol., vol. 85, 1993, 133-142). As shown in Fig. 4, the amount of Factor VIII cofactor activity in medium is increased in time in the absence of RAP. In the presence of RAP, however, the extent of Factor VIII rise is increased compared to the absence of RAP. Thus, inhibition of LRP is associated with an accumulation of Factor VIII in medium. It is evident therefore that the cellular uptake of Factor VIII light chain involves a LRP-dependent mechanism.

Example V: The effect of RAP on the Factor VIII pharmacokinetics in knock-out mice with severe Factor VIII-deficiency.

A genetically engineered mouse strain with severe Factor VIII (FVIII)-deficiency was made by targeted disruption of the mouse Factor VIII-gene, according to Bi et al., Nature Genetics, 1995, vol. 10, pp. 119-121. Factor VIII knock-out mice were created by an insertion of a neo-gene into the 3´-end of exon 17 of the mouse Factor VIII gene. The affected males (X´Y) had undetectable Factor VIII-levels of $< 0.02 \pm 0.01$ U/ml when measured either with a chromogenic Factor VIII assay, Hyland Immuno, Vienna, Austria, as described recently (Turecek et al., Thromb. Haemostas. Suppl., 1997, vol. 769) or by antigen ELISA as described below.

Two hemizygous affected male mice (X´Y) were treated with a recombinant human Factor VIII (rhFVIII) preparation derived from Chinese hamster ovary cells produced as described (WO 85/01961) and pharmaceutically formulated without any stabilizing protein at a dose of 200 U/kg body weight given intravenously.

Under anesthesia, one hour after treatment, the tail tips of the mice were cut by a scalpel blade as described by Novak et al. (Brit. J. Hematol., vol. 69, 1988, pp. 371-378). A volume of 50µl blood was collected from the tail wounds with capillaries (Ringcaps, Hirschmann, Germany) which capillaries were coated with lithium heparin as anticoagulant. The capillaries were closed and centrifuged to separate blood cells from plasma. Subsequently, the capillaries were opened and the cell and the plasma fraction were collected by further centrifugation. Finally, the plasma samples were applied to Factor VIII determination by the Factor VIII antigen ELISA, test kit IMMUNOZYM FVIII Ag, Hyland Immuno, Vienna, Austria, using monoclonal anti-factor VIII antibodies both for capture and detection as described (Stel et al., Nature, 1983, vol. 303, pp. 530-532; Lenting et al., J. Biol. Chem., vol. 269, 1994, pp. 7150-7155; Leyte et al., Biochem. J., vol. 263, 1989, pp. 187-194). The resulting Factor VIII-values were expressed in international units of human Factor VIII. The results of Factor VIII plasma levels are outlined in the table.

Two other hemizygous affected male mice (X´Y) were pretreated with recombinant receptor associated protein (GST-RAP) 10

minutes prior to the treatment with the recombinant human Factor VIII, at a dose of 40 mg/kg body weight. The RAP used in this study, which interacts with LRP, was obtained by bacterial fermentation as described by Herz et al. (J. Biol. Chem., vol. 266, 1991, pp. 21232-21238). A fusion protein of the RAP with glutathione S-transferase was expressed in an E. coli and purified by affinity chromatography on glutathione agarose. The resulting protein mainly consisted of the fusion protein and split products of RAP and glutathione S-transferase. The fusion protein was formulated in an injectable buffer ready for administration to the Factor VIII knock-out mice. As in the control group (treatment with Factor VIII alone), blood samples were obtained one hour after the administration of recombinant Factor VIII and measured for active Factor VIII using the Factor VIII antigen ELISA.

The results are outlined in the table:

	treatment dose	treatment dose	recovery 1 h post treatment
mouse no.	GST-RAP	rhFVIII	FVIII:Ag (U/ml plasma)
1	40 mg/kg	200 U/kg	1.92
2	40 mg/kg	200 U/kg	1.88
3		200 U/kg	0.73
4		200 U/kg	0.83

Factor VIII level in mice pretreated with GST-RAP were more than 200% of the plasma levels following treatment with recombinant Factor VIII alone. The administration of an LRP antagonist, RAP, improved the pharmacokinetics of Factor VIII.

Example VI: Identification of potential LRP-binding exosites on the Factor VIII light chain.

A method for the identification of exosites potentially involved in protein-protein interaction has been previously established (J. Kyte and R.F. Doolittle, J. Mol. Biol. Vol. 157, 1982, pp. 105-132). This method provides a program that progressively

evaluates the hydrophilicity and hydrophobicity of a protein along its amino acid sequence. The method employs a hydropathy scale which reflects the average hydropathy within segments of predetermined size along the amino acid sequence. Hydrophilic sections are characterised by negative hydropathy values, and are likely candidates to be oriented towards the exterior of a protein which is in an aqueous solution. This method has been applied to the known sequence of human Factor VIII (G.A. Vehar et al., Nature, vol 312, 1984, pp. 337-342; J.J. Toole et al., Nature, vol 312, 1984, 342-347), employing a segment size ("window") of 19 residues. From the complete sequence of Factor VIII the region 1690-2332, which corresponds with the Factor VIII A3/C1/C2-domain, was subjected to this analysis, and the resulting hydropathy plot, having a value of -15 as a cut off is shown in Figures 5A and 5B.

This method revealed several discrete regions with low hydropathy values, which reflect the hydrophilic nature associated with potential exosites. These are indicated as A to K (Table IV):

Table IV:

Site	Residues	Domain
A	Met 1711 to Gly 1725	A3
B	Phe 1743 to Arg 1749	A3
C	Ser 1784 to Asp 1831	A3
D	Ser 1888 to His 1919	A3
E	Trp 1942 to Met 1947	A3
F	Ser 1959 to Ala 1974	A3
G	Ile 2037 to Trp 2062	C1
H	Asp 2108 to Asn 2118	C1
I	Thr 2154 to Ile 2158	C1
J	Arg 2209 to Phe 2234	C2
K	His 2269 to Lys 2281	C2

Table V:

Site	Residues	Domain
I	Phe 1785 to His 1822	A3
II	Trp 1889 to Asn 1915	A3
III	Trp 2112 to Tyr 2115	C1
IV	His 2211 to Leu 2230	C2

Again, from the complete sequence of Factor VIII the region 1690-2332, which corresponds with the complete Factor VIII light chain, was subjected to this analysis, and the resulting hydropathy plot, having a value of -20 as a cut off is shown in Figure 5b. The exosites are indicated as I to IV.

Example VII: The Factor VIII C2-domain comprises a LRP binding site

The A3-C1-C2 region of Factor VIII encompasses the binding moiety for LRP (see Example I). This region comprises a number of potentially LRP-binding exosites in its constituent domains, including in the C2-domain (see Example VI). To confirm that such exosites indeed may be involved in LRP binding, the interaction between LRP and the Factor VIII C2-domain was analysed in more detail. Factor VIII C2-domain (*i.e.* residues 2172-2332) was expressed in insect cells employing a previously established method (K. Fijnvandraat et al., Blood vol. 91, 1998, pp. 2347-2352). Factor VIII C2-domain was purified by immunoaffinity chromatography employing the C2-domain directed monoclonal antibody CLB-CAg 117 (K. Fijnvandraat et al., Blood, Vol. 91, 1998, pp. 2347-2352). The interaction with LRP was examined employing surface plasmon resonance analysis at a BIAcore™2000 system (Pharmacia Biosensor AB, Uppsala, Sweden). LRP was immobilized onto a CM5 sensorchip as described in Example I. To enhance the resonance signal, Factor VIII C2-domain (0, 100 or 375 nM) was pre-incubated in the presence of 500 nM of the C2-domain directed monoclonal antibody ESH-8 (D. Scandella et al., Blood, vol. 86, 1995, pp. 1811-1819) in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, 0.005 % (v/v) Tween-20 for 15 minutes at room temperature. Subsequently, pre-incubated

samples were passed over the control channel to asses non-specific binding and over the LRP-coated channel (8.3 fmol/mm^2) with a flow of 5 ml/min for 2 min at 25°C.

In the absence of C2-domain, ESH-8 displays minimal binding, if any, to immobilized LRP. In the presence of C2-domain, however, a dose-dependent increase in binding to LRP was observed (Fig. 6). This demonstrates that the Factor VIII C2-domain binds to LRP. Thus, exosites within the Factor VIII light chain indeed have the potential of LRP binding, and thus to be involved in LRP dependent clearance of Factor VIII in vivo.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The compounds, methods and compositions described herein are presented as representative of the preferred embodiments. These are intended to be exemplary and not as limitations on the scope of the present invention. Modifications thereof and other uses will occur to those skilled in the art, and are to be included within the spirit of the invention and the scope of the appended claims.

Claims:

1. A Factor VIII polypeptide with Factor VIII:C activity having a modification in the A3 and/or C1 and/or C2 domain of the light chain characterized in that the modification affects the binding affinity to Low Density Lipoprotein Receptor Protein (LRP).

2. A Factor VIII polypeptide according to claim 1 characterized in that the modification is between AA 1690 and 2032, AA 2033 and 2172 and/or AA 2173 and 2332.

3. A Factor VIII polypeptide according to claims 1 and 2 characterized in that the modification is between AA 1711 (Met) and 1725 (Gly), AA 1743 (Phe) and 1749 (Arg), AA 1888 (Ser) and 1919 (His), AA 1942 (Trp) and 1947 (Met) and/or AA 1959 (Ser) and 1974 (Ala).

4. A Factor VIII polypeptide according to claims 1 and 2 characterized in that the modification is between AA 2037 (Ile) and 2062 (Trp), AA 2108 (Asp) and 2118 (Asn) and/or AA 2154 (Thr) and 2158 (Ile).

5. A Factor VIII polypeptide according to claims 1 and 2 characterized in that the modification is between AA 2209 (Arg) and 2234 (Phe) and/or AA 2269 (His) and 2281 (Lys).

6. A Factor VIII polypeptide according to claim 5 characterized in that the modification is between AA 2112 (Trp) and 2115 (Tyr).

7. A Factor VIII polypeptide according to claim 5 characterized in that the modification is between AA 2211 (His) and 2230 (Leu).

8. A DNA molecule coding for a Factor VIII polypeptide with Factor VIII:C activity having a modification in the A3, C1 and/or C2 domain of the light chain characterized in that the modification affects the binding affinity to Low Density

Lipoprotein Receptor Protein (LRP).

9. A DNA molecule coding for a Factor VIII polypeptide according to claim 8 characterized in that the modification is between AA 1690 and 2032, AA 2033 and 2172 and/or AA 2173 and 2332.

10. A DNA molecule coding for a Factor VIII polypeptide according to claims 8 and 9 characterized in that the modification is between AA 1711 (Met) and 1725 (Gly), AA 1743 (Phe) and 1749 (Arg), AA 1888 (Ser) and 1919 (His), AA 1942 (Trp) and 1947 (Met) and/or AA 1959 (Ser) and 1974 (Ala).

11. A DNA molecule coding for a Factor VIII polypeptide according to claims 8 and 9 characterized in that the modification is between AA 2037 (Ile) and 2062 (Trp), AA 2108 (Asp) and 2118 (Asn) and/or AA 2154 (Thr) and 2158 (Ile).

12. A DNA molecule coding for a Factor VIII polypeptide according to claims 8 and 9 characterized in that the modification is between AA 2209 (Arg) and 2234 (Phe) and/or AA 2269 (His) and 2281 (Lys).

13. A DNA molecule coding for a Factor VIII polypeptide according to claim 11 characterized in that the modification is between AA 2112 (Trp) and 2115 (Tyr).

14. A DNA molecule coding for a Factor VIII polypeptide according to claim 12 characterized in that the modification is between AA 2211 (His) and 2230 (Leu).

15. An expression vector comprising a DNA molecule according to any one of claims 8 to 14.

16. A transformed cell and progeny thereof comprising a DNA molecule according to any one of claims 8 to 14.

17. A method for the production of a Factor VIII polypeptide with Factor VIII:C activity having a modification in the A3 and/or C1 and/or C2 domain of the light chain sequence

characterized in that the modification affects binding affinity to LRP, said method comprising:
- growing, in a nutrient medium, a host cell comprising an expression vector comprising, in the direction of transcription, a transcriptional regulatory region and a translational initiation region functional in a host cell,
- a DNA sequence encoding for a polypeptide as defined in any one of claims 1 to 7 and
- translational and transcriptional termination regions functional in said host cell,
wherein expression of said DNA sequence is regulated by said initiation and termination regions, and
- isolating said polypeptide.

18. A preparation comprising a FVIII molecule having FVIII:C activity and a polypeptide selected from the group of LRP antagonists.

19. A preparation according to claim 18 characterized in that the FVIII molecule is a recombinant Factor VIII polypeptide with FVIII:C activity having a modification in the A3, C1 and/or C2 domain of the light chain characterized in that the modification affects the binding affinity to LRP.

20. A preparation according to claim 18 characterized in that that LRP antagonist selected from the group of RAP and soluble fragments of LRP.

21. A preparation according to claim 18 characterized in that the soluble fragments of LRP show binding affinity to the FVIII LRP binding site.

22. Use of a Factor VIII polypeptide according to any one of claims 1 to 7 or a preparation according to any one of claims 18 to 21 for the formulation of a preparation for the treatment of a coagulation disorder.

23. Use of a preparation according to claim 22 wherein the coagulation disorder is hemophilia A.

Summary:

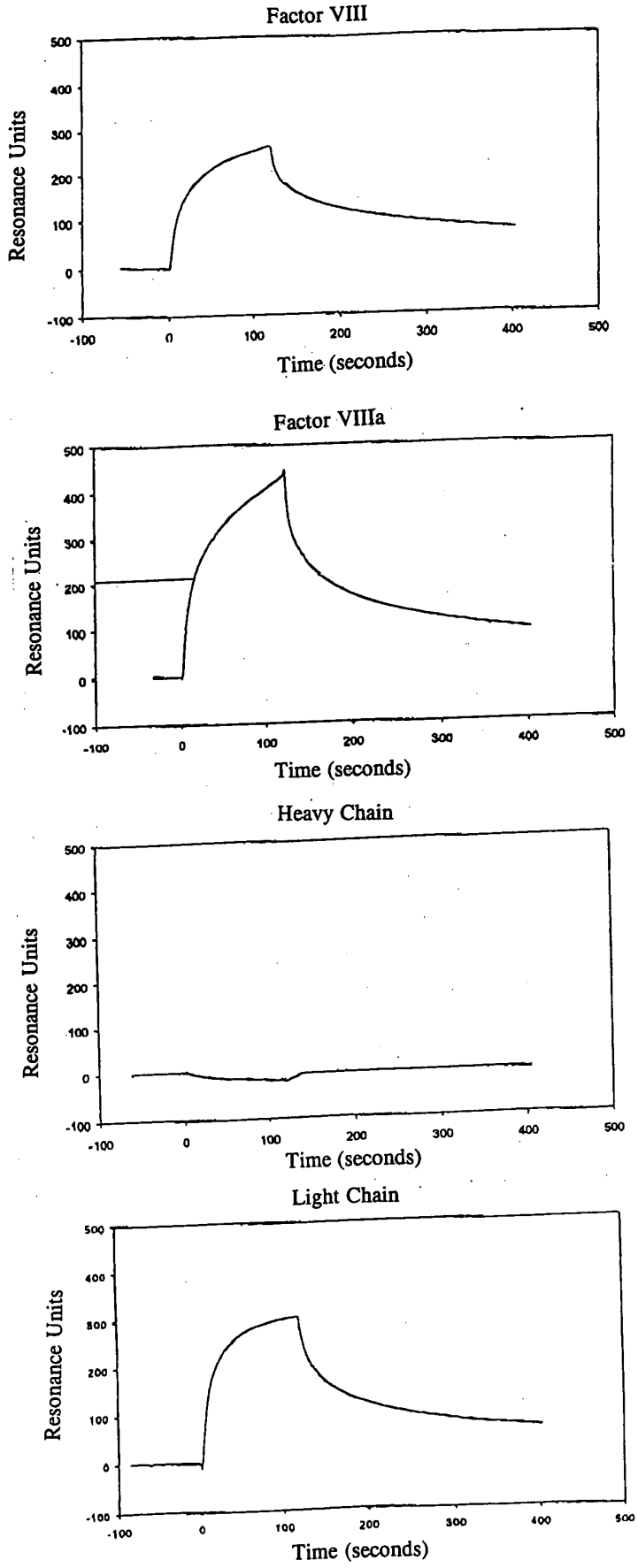
This invention describes a Factor VIII polypeptide with Factor VIII:C activity having a modification in the A3 and/or C1 and/or C2 domain of the light chain, which is characterized in that the modification affects the binding affinity to Low Density Lipoprotein Receptor Protein (LRP) and a method for the production thereof.

Fig. 4

10. Nov. 1998

Urtext

FIG. 1


Factor VIII
Resonance Units
Time (seconds)
Factor VIIIa
Resonance Units
Time (seconds)
Heavy Chain
Resonance Units
Time (seconds)
Light Chain
Resonance Units
Time (seconds)

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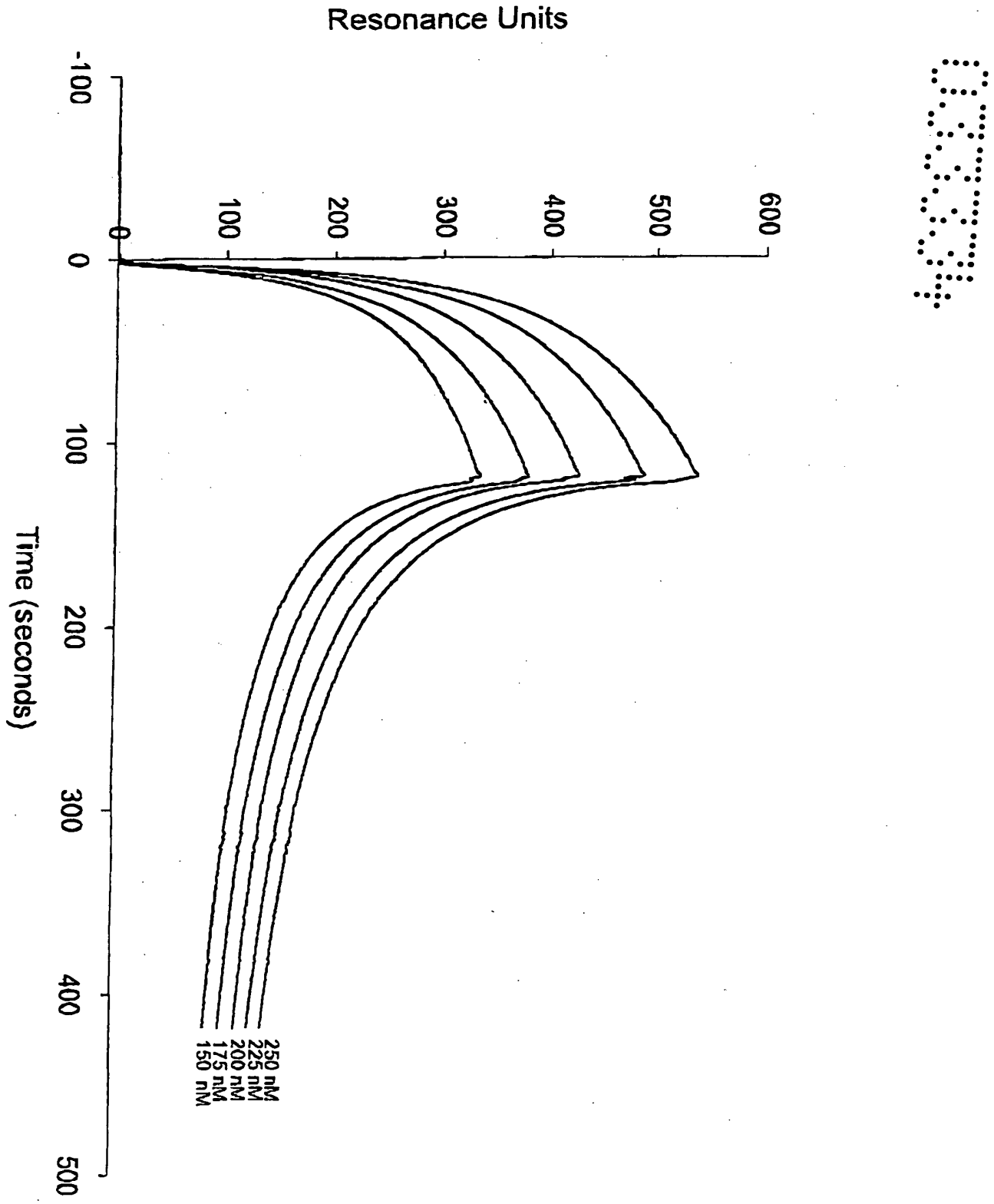
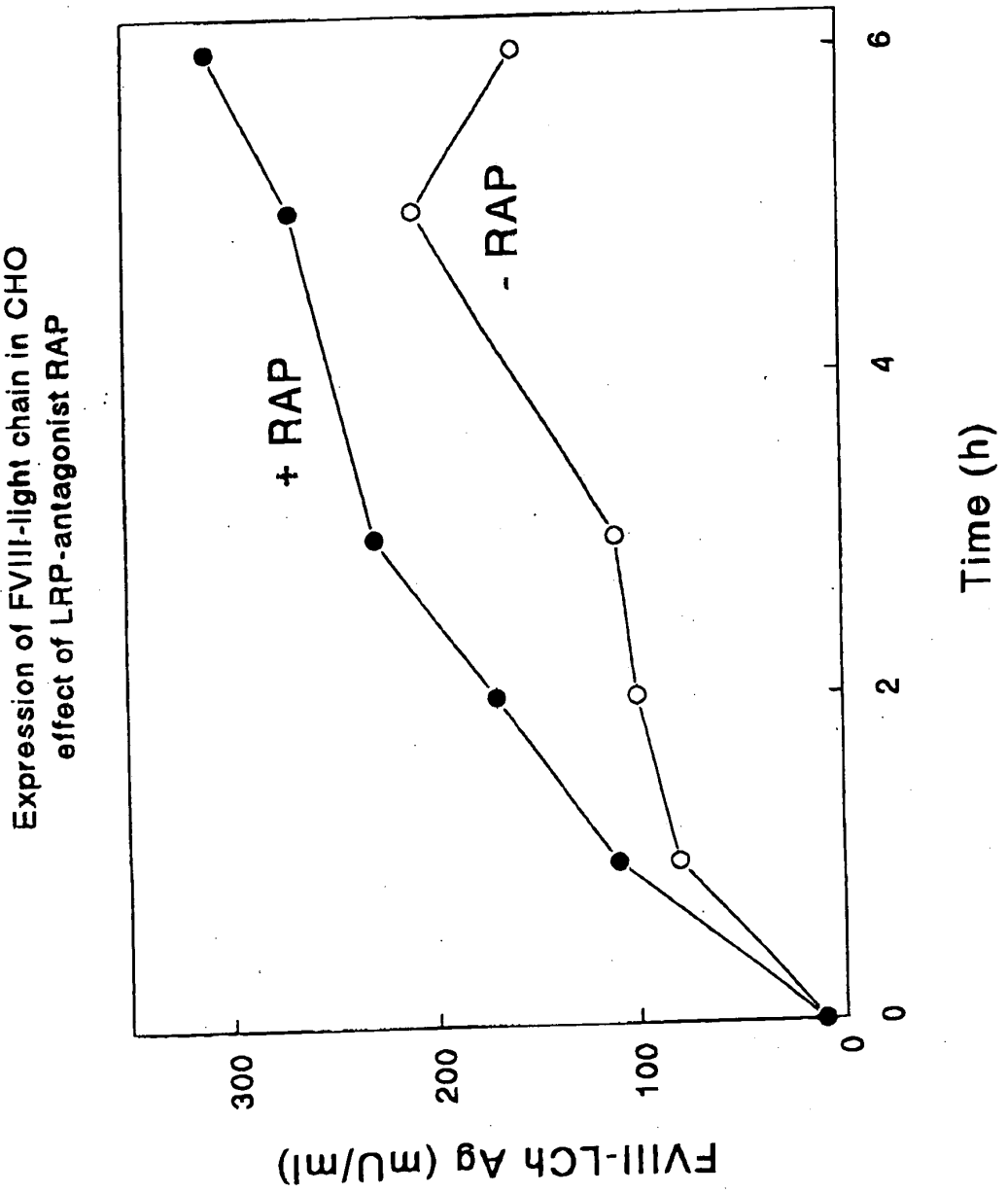
Urtext

FIG. 2



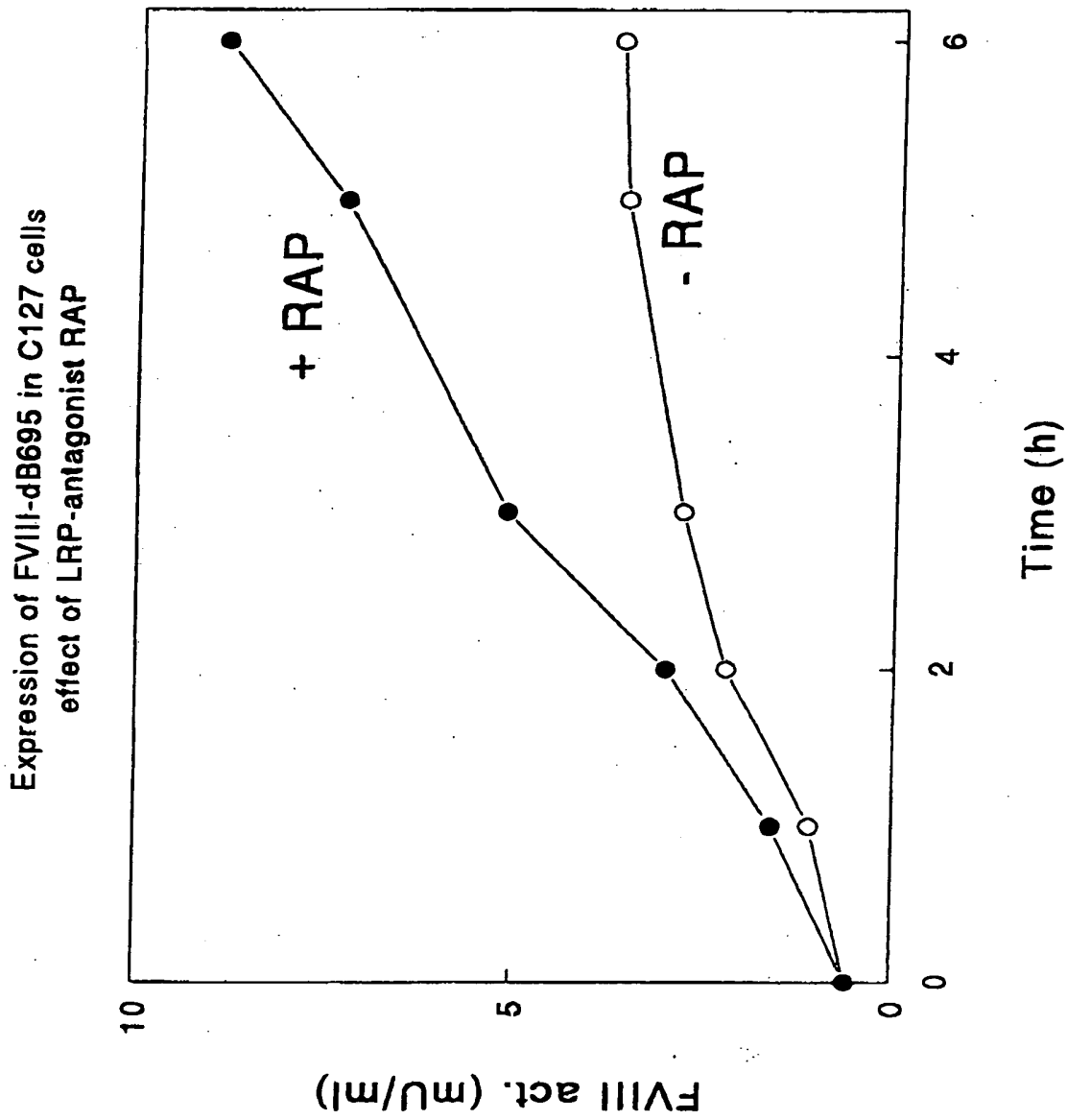
Urtext

FIG. 3


Expression of FVIII-light chain in CHO
effect of LRP-antagonist RAP
+ RAP
- RAP
FVIII-LCh Ag (mU/ml)
Time (h)
0
100
200
300
0
2
4
6

Uftext

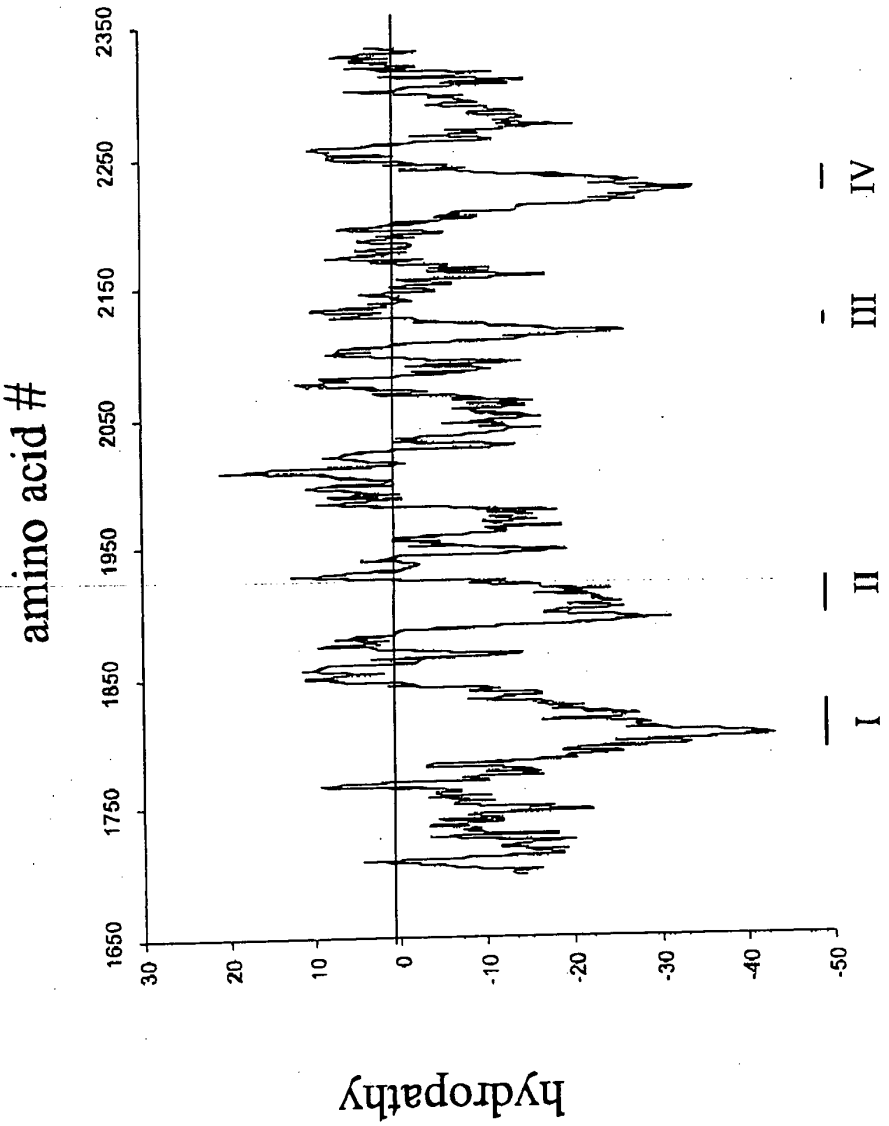
FIG. 4


Expression of FVIII-dB695 in C127 cells
effect of LRP-antagonist RAP
+ RAP
- RAP
FVIII act. (mU/ml)
Time (h)
0
5
10
0
2
4
6

A1872/98-1

Artext

FIG. 5A



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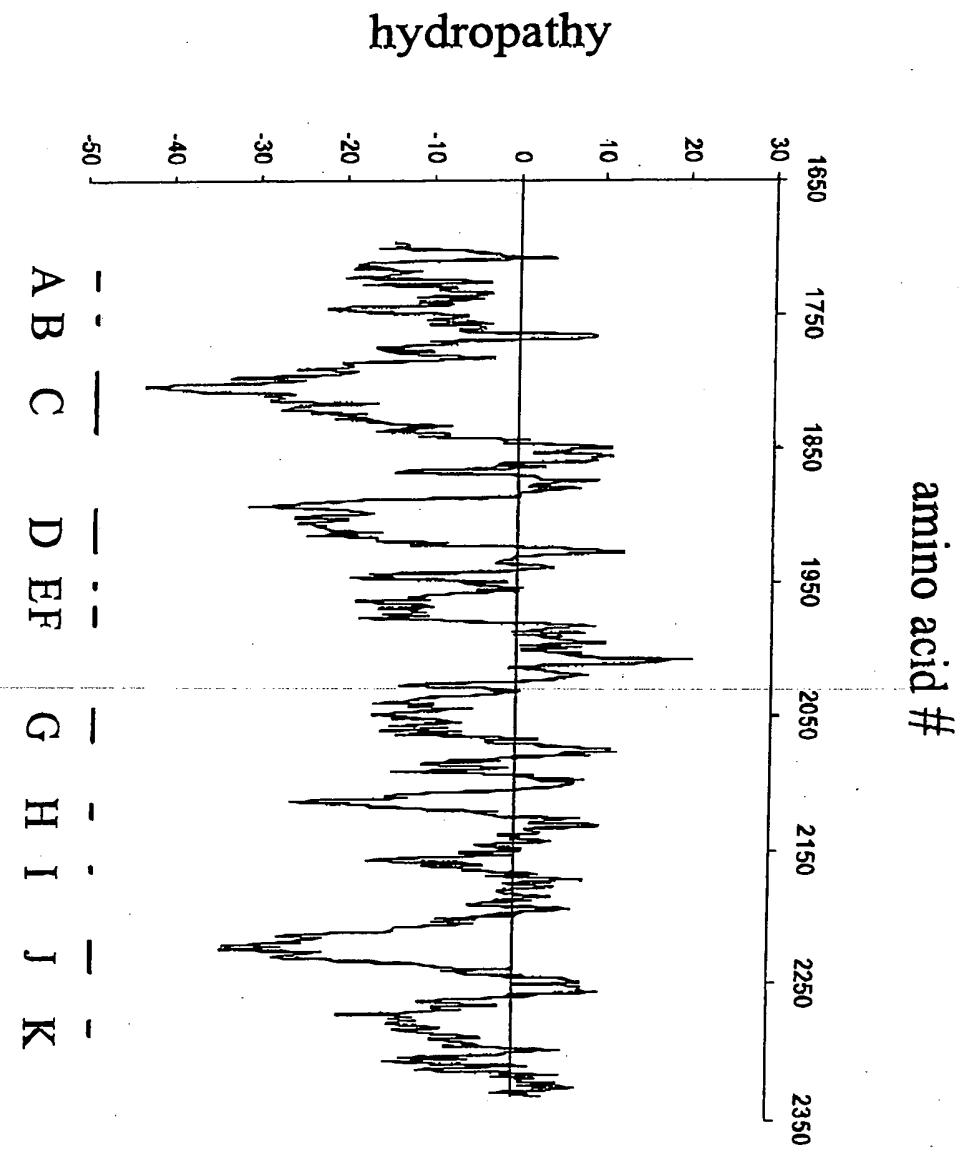
Urtext

FIG. 5B



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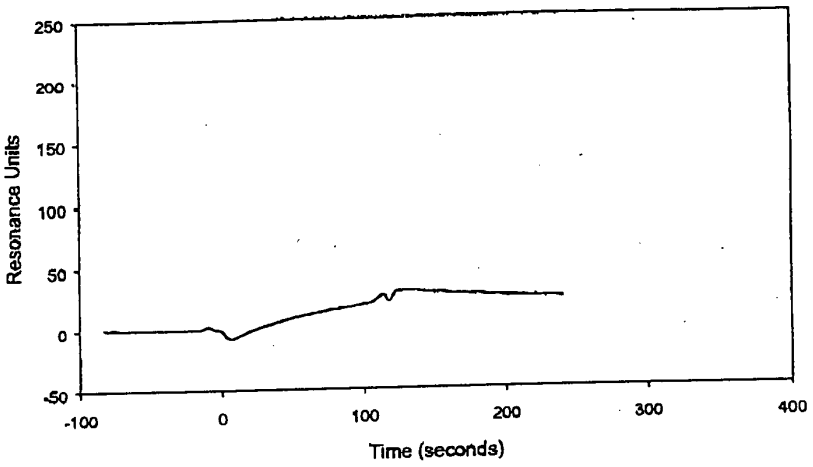
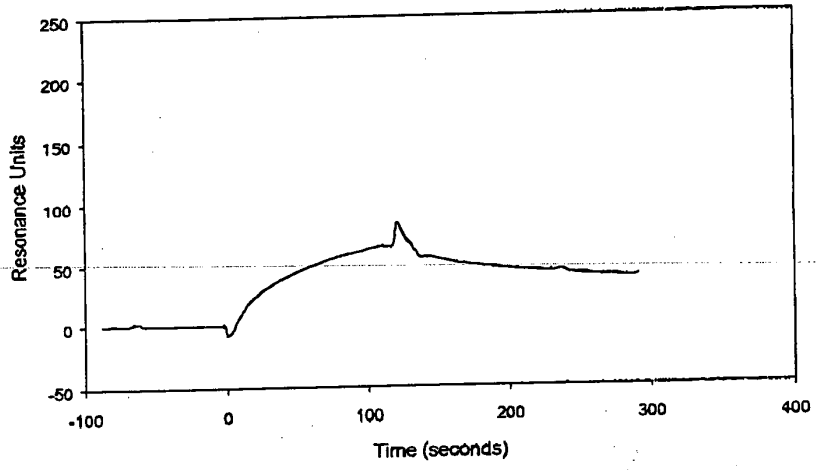
Urtext

FIG. 6

500 nM ESH8



100 nM C2 + 500 nM ESH8



370 nM C2 + 500 nM ESH8